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In mice, ectopic activation of certain wnt genes, such as wnt-1, causes the formation of mammary tumors, providing a potential model for human breast cancer. However, Wnt receptors, essential components mediating the Wnt oncogenic function, had not been identified. It was shown recently that in *Drosophila*, DFz2, a member of the Frizzled (Fz) family of seven transmembrane receptors, functions as a receptor for Wingless protein, the ortholog of Wnt-1. This implies that the family of Fz proteins are likely to function as receptors for Wnt molecules. However, the scarcity of soluble Wnt proteins complicates the study of ligand-receptor relationships and their specificity. We have developed an approach in *Xenopus* to pursue the study.

In *Xenopus*, Wnt molecules of Wingless/Wnt-1 subclass induce axis duplication whereas Wnt-5A subclass molecules do not. This dichotomy can be explained by activation of different signaling pathway(s), or lack of Wnt-5A receptor(s) during axis formation. We found that Wnt-5A induces axis duplication in the presence of hFz5, a member of the Frizzled protein family. Wnt-5A signaling via hFz5 is antagonized by glycogen synthase kinase-3 (GSK-3) and the N-terminal ectodomain of hFz5. These results identifies hFz5 as a receptor for Wnt-5A, and establishes axis induction in *Xenopus* as an approach to investigate relationships between Wnt and Fz proteins.

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INTRODUCTION

Wnt genes encode a large family of secreted signaling molecules essential for development and tumorigenesis (1, 2). Of particular interests to human breast cancer research, ectopic activation of certain wnt genes, such as wnt-1, causes mammary carcinogenesis in mice (3). How Wnt-1 induces mammary tumors is not understood. However, given that Wnt molecules are secreted factors, it is likely that a receptor- mediated signaling transduction pathway is involved. The aim of this project has been to identify Wnt receptor(s).

In *Drosophila ,wingless (wg)* gene, the ortholog of murine wnt-1, is required for many stages of embryogenesis (4, 5). Interestingly, upon gene transfer *wg* cDNA can transform murine mammary epithelial cells as does wnt-1 cDNA, demonstrating a striking functional conservation between *wg* and wnt-1(6). Recently, a receptor for Wg was identified to be a member of the *Drosophila* Frizzled (Fz, ref. 7) family of seven transmembrane receptors. DFz2, when introduced by transfection, confers recipient cells the ability to bind and to respond to Wg (8). The identification of DFz2 as a Wg receptor implies that the fz family of genes, which include eight mammalian members identified so far (9, 10), are likely to encode receptors for the Wnt family. However, the scarcity of soluble Wnt proteins (11) complicates the investigation of Wnt-Fz relationships. We have developed an approach to address this issue using *Xenopus* embryos without soluble Wnt proteins.

In *Xenopus*, several Wnt molecules, such as Wg, Wnt-1, the *Xenopus* wnt-3A and wnt-8 (Xwnt-8), have been shown to mimic an early dorsalizing signal that induces the gastrula organizer and in doing so are able to induce embryonic axis duplication (12-17), thus offering a unique opportunity to study Wnt function and Wnt signal transduction pathway. However, several other Wnt proteins, such as the *Xenopus* Wnt-5A, were found unable to induce axis duplication, and have been classified as a

distinct subclass that activates a different pathway(s) (17-19). In this report we show that Wnt-5A can induce axis duplication in the presence of a member of the mammalian Fz family, hFz5, but not other Fz proteins tested. Wnt-5A signaling via hFz5 is antagonized by the hFz5 N-terminal ectodomain that presumably binds and titrates Wnt-5A. These results identify hFz5 as a receptor for Wnt-5A. Importantly, because Wnt-5A is expressed during the development of mammary gland (20), the revelation of Wnt-5A-hFz5 ligand-receptor relationship will enhance the understanding of normal mammary development and physiology. Moreover, this study establishes axis induction in *Xenopus* as an approach to investigate relationships between Wnt and Fz proteins in general, and to identify receptors for oncogenic Wnt molecules in mammary tumors in particular.

RESULTS AND DISCUSSION

Mouse Wnt-1, Xwnt-8, and *Drosophila* Wg are capable of inducing dorsal development when low levels, usually 1-10 pg, of their corresponding RNAs are injected into the ventral side of early *Xenopus* embryos (12-15, and data not shown). In contrast, *Xenopus* wnt-5A (Xwnt-5A) RNA fails to do so even after ventral injection at higher doses (75pg to 1ng per embryo); instead, dorsal injection of Xwnt-5A RNA generates head and tail defects that may result from perturbation of cell movement during gastrulation (18). Xwnt-4 and Xwnt-11 behave similarly to Xwnt-5A (19). The Xwnt-8 dorsalizing function is observed before the mid-blastrula transition (MBT), i.e., before the start of zygotic transcription, whereas the Xwnt-5A effect occurs after MBT (13-15, 17). The difference between the consequences of injecting Xwnt-8 and Xwnt-5A RNAs may reflect the activation of distinct signaling pathways or, alternatively, the lack of a functional Xwnt-5A receptor that can couple to the dorsalizing machinery in the early embryos. The latter possibility predicts that Xwnt-5A might function as a dorsalizing signal if its receptor(s) were provided experimentally to the early embryo.

To examine whether a particular Fz protein can function as an Xwnt-5A receptor, synthetic RNAs corresponding to Dfz2 (ref. 8) and six mammalian fz cDNAs, mfz3, 4, 6, 7, 8 (from mouse) and hfz5 (from human, ref. 10), were pooled into two groups and co-injected with 10 pg Xwnt-5A RNA into the ventral side of 4-cell stage embryos. Injection of Xwnt-5A alone, either fz group alone, or Xwnt-5A together with the fz group 2 (mfz3, 4, 6, and 7) produced no phenotypic effects (Fig. 1a). However, co-injection of Xwnt-5A with the fz group 1 RNAs (Dfz2, hfz5 and mfz8) induced extensive dorsal axis duplication; in many cases, duplication was complete as

determined by the presence of anterior structures such as the eyes and the cement gland (Fig.1a and 3a). When the three fz RNAs in Group 1 were independently tested, we observed that Xwnt-5A plus hfz5 generated axis duplications, whereas Xwnt-5A and Dfz2 or mfz8 did not (Fig. 1b). Thus, hFz5 alone among the Fz proteins tested is responsible for mediating axis induction by Xwnt-5A. The mature Wnt-5A proteins (after cleavage of the signal peptides) are 100% identical between mouse and human, and 95% identital between mouse and *Xenopus* (18, 21, 22). Not surprisingly, murine wnt-5A RNA also induced axis duplication when co-injected with hfz5 RNA, albeit less efficiently (Fig. 1c). One possible explanation might be that the presence of 5' and 3' untranslated regions in the wnt-5A construct reduced RNA stability and/or translation efficiency. Dorsal injection of the same concentration of Xwnt-5A plus hfz5 RNAs produced no axis duplication and injected embryos appeared normal (not shown).

Axis duplication by Xwnt-5A plus fz group 1 or hfz5, as described above, was observed in fourteen of twenty embryo batches tested. In these cases, 90-100% of the injected embryos showed axis duplication, of which 17-82% were complete. In the remaining six of twenty embryo batches, the same co-injection induced no or a few axis duplications (less than 30%, none complete). The reason for this poor response in some embryo batches are unknown. Possible explanations might include variations in the stability of injected RNAs and/or translated proteins, the efficiency of Wnt-5A secretion, the assembly and/or localization of hFz5 protein, or the availablity of unknown co-receptor molecules.

It should be noted while hFz5 is more closely related to DFz2 and mFz8 than to any other known Fz proteins, neither Dfz2 nor mFz8 cooperated with Xwnt-5A in axis induction. However, DFz2 appeared to be functional in *Xenopus* embryos since the same concentration of Dfz2 RNA (as used for co-injection with Xwnt-5A) substantially enhanced axis induction by suboptimal amounts of *wg* RNA (not shown). A dose response curve illustrated that in the presence of 0.4 ng

hfz5 RNA per embryo, 1 pg Xwnt-5A RNA induced partial secondary axes whereas 20 pg of Xwnt-5A RNA sometimes hyperdorsalized embryos (Fig. 2). Although the relative protein levels have not been determined, the dose of Xwnt-5A RNA required for axis induction was in a similar range to effective doses of Xwnt-3A, Xwnt-8, wnt-1 and *wg* RNAs. Together these data suggest a high degree of specificity of the interaction between Xwnt-5A and hFz5.

Histological examination of embryos with duplicated axes revealed that Xwnt-5A and hFz5 induced a full array of dorsal tissues, including notochord, neural tube and somites (Fig.3b). There is one notable difference between axes induced by Xwnt-8 and those induced by Xwnt-5A plus hFz5: while the ectopic axes induced by Xwnt-8 are often indistinguishable from the endogenous ones, the axes induced by Xwnt-5A and hFz5 are shorter in most cases, even when the eyes and the cement gland are present. This might reflect the previously described ability of Xwnt-5A to block cell movement during gastrulation (18).

Xwnt-8 and other Wnt proteins in its group induce dorsal development via the formation of an ectopic Spemann organizer (13-15). We tested whether Xwnt-5A plus hFz5 act similarly by examining the expression of the organizer-specific gene *goosecoid* (*gsc*, ref. 23), using wholemount *in situ* hybridization. As shown in Figure 3c and consistent with a previous study on Xwnt-5A (18), embryos injected with Xwnt-5A or hfz5 RNA alone expressed *gsc* only dorsally, as did uninjected controls; in contrast, embryos co-injected with Xwnt-5A and hfz5 RNAs exhibited two opposing centers of *gsc* expression, indicating the ectopic formation of a secondary organizer.

In *Drosophila*, Wg function is mediated by inhibition of the *zeste-white 3 (shaggy)* gene products, the homolog of the vertebrate glycogen synthase kinase-3 (GSK-3) (24). Likewise, the dorsalizing function of Xwnt-8 RNA in *Xenopus* embryos is mimicked by dominant-negative

mutant forms of GSK-3 and antagonized by overexpression of wild type GSK-3 (25-27). We therefore asked whether Xwnt-5A signaling via hFz5 is also transduced by inhibition of GSK-3. As shown in Figure 4, co-injection of wild type GSK-3β RNA completely blocked the dorsal axis duplication by Xwnt-5A and hFz5, consistent with the notion that, just as in Xwnt-8 signaling, Xwnt-5A signaling via hFz5 requires the inhibition of GSK-3.

All Fz proteins contain a putative N-terminal extracellular domain composed of a conserved cysteine-rich region (CRD) and a variable linker region before the first putative transmembrane helix (10). The N-terminal ectodomain of DFz2, when anchored to the membrane, was sufficient to promote Wg binding to the cell surface, and deletion of CRD from the ectodomain of mFz4 abolished Wg binding (8). Thus the N-terminal extracellular domain appears necessary and sufficient for ligand binding. We reasoned that if the N-terminal ectodomain of hFz5 binds Xwnt-5A, overexpression of this domain as a secretory molecule should titrate the available Xwnt-5A and prevent Xwnt-5A signaling through hFz5. As shown in Fig. 5, co-injection of RNA coding for the hFz5 N-terminus (hFz5N), without provision of a membrane anchor, antagonized axis induction by Xwnt-5A plus hFz5 in a dose-dependent manner, with 40% to 80% inhibition achieved with 400 and 800 pg hfz5N RNA per embryo in the presence of 400 pg hfz5 RNA. A higher dose of hfz5N RNA (2 ng per embryo) resulted in almost complete suppression of axis duplication, although abnormal embryonic development (possibly due to effects on gastrulation) was frequently noticed. As a control, dorsal injection of 800 pg or 2 ng of hfz5N RNA did not affect endogenous dorsal axis formation (although gastrulation defects were seen in some embryos); the injected embryos appeared to have an intact neural groove at the neurula stage and the anteriormost structures (eyes and the cement gland) at the tadpole stage (data not shown). These results argue for a specific effect of hFz5N on blocking dorsal axis duplication by Xwnt-5A plus hFz5.

CONCLUSIONS

Our data indicate that in the presence of hFz5, Wnt-5A proteins can transduce a signal similar to that of Xwnt-8. This signal appears to be mediated by a common pathway involving down-regulation of GSK-3 activity. The simpliest interpretation of these results is that hFz5 functions as a receptor for amphibian and mammalian Wnt-5A, thus extending the ligand-receptor relationships of Wnt and Fz families to vertebrates. Given the high degree sequence identity between mature Wnt-5A proteins across species, these data suggest that human Fz5 is likely to be a receptor for human Wnt-5A.

At least fourteen wnt and eight fz genes have been identified in mammals thus far. The ligand-receptor relationships among Wnt and Fz proteins are likely to be complex, as seen in other large families of signaling molecules and their receptors. Biochemical study of the Wnt-Fz interaction remains challenging because of the difficulty in obtaining soluble Wnt proteins. By reconstituting a Wnt-Fz signaling system in *Xenopus* embryos and blocking this signaling system via a dominant-negative form of Fz ectodomain, the experiments described here provide a general assay to address the relationships among Wnt and Fz proteins, and to identify Fz proteins as receptors mediating oncogenic function of Wnt molecules in mammary tumorigenesis.

FIGURE LEGENDS

Figure 1. Dorsal axis induction by Xwnt-5A mediated by hFz5. In all the histograms of this and the following figures, n represents the total number of embryos scored from two to six independent experiments; each bar represents the percentage of axis duplication; the solid portion within each bar represents the percentage of complete axis duplication, which is defined by the presence of the cement gland and at least one eye in the duplicated axis. Unless otherwise specified, 10 pg of Xwnt-5A RNA and/or 400 pg of each *fz* RNAwere injected per embryo. **a).** Ventral injection of Xwnt-5A RNA together with RNA for *fz* group 1 (Dfz2, hfz5 and mfz8) induces axis duplication. Xwnt-5A RNA alone, *fz* group 1 or group 2 (mfz3, 4, 6, and 7) alone, or Xwnt-5A RNA plus *fz* group 2 did not. **b).** Xwnt-5A RNA induces axis duplication in the presence of hfz5 RNA, but not Dfz2 or mfz8 RNA. c) Murine wnt-5A RNA (10 pg per embryo) also induces axis duplication with hfz5.

Figure 2. Dose response curve of Xwnt-5A and hfz5 RNAs for axis duplication.

Figure 3. Xwnt-5A plus hfz5 induction of anteriormost structures, of dorsal axial tissues, and of the Spemann organizer. **a).** Xwnt-5A plus hfz5 induce axis duplication. Shown here is an example of complete axis duplication with eyes in both axes, photographed at stage 41. **b).** Xwnt-5A plus hfz5 induce a complete set of dorsal tissues. Cross section in the trunk region of a stage 41 reveals the presence of a neural tube, a notochord and somites in both axes. **c).** Xwnt-5A plus hfz5 induce ectopic *goosecoid (gsc)* expression in stage 11 embryos, as visualized by whole mount *in situ* hybridization.

Figure 4. GSK-3β antagonize axis induction by Xwnt-5A and hfz5. RNA for human GSK-3β was injected at 1ng per embryo. **a).** Examples of control (uninjected) and injected embryos at stage 19. **b).** Note the complete suppression of axis duplication in the presence of GSK-3β.

Figure 5. The N-terminal ectodomain of hFz5 (hFz5N) as a secreted protein suppresses axis duplication by Xwnt-5A plus hfz5 in a dose dependent manner. Dorsal injection of same doses of hfz5N RNA did not affect the endogenous dorsal axis (data not shown).

METHODS

Plasmid constructs

All fz cDNAs were subcloned in the pRK5 vector with a optimal Kozak consensus sequence for translation at the initiator ATG (CCACCATG, preceeded by different restriction sites for subcloning), and with different lengths of 3' untranslated regions. Xwnt-5A and human GSK-3β were in pSP64 vector as described (18, 26). Murine wnt-5 cDNA (ref. 21) was cloned in pCS2+ vector as the EcoRI and XbaI fragment, which contains about 60 bp 5' and 360 bp 3' untranslated regions. hfz5N was generated by introduction of a STOP codon just before the first putative transmembrane helix (changing amino acids 237 and 238 from phenylalanine and trytophan to threonine and arginine followed by a STOP codon), and the corresponding DNA fragment was subcloned in pCS2+.

RNA injection

All RNAs for injection were synthesized as capped transcripts *in vitro* with SP6 RNA polymerase (Ambion Megascript). Unless otherwise specified, RNAs were injected into the two ventral cells near the equatorial midline at the 4-cell stage.

Embryo handling

Embryo preparation, staging, fixation and sectioning, and wholemount *in situ* hybridization were performed as described (26).

Scoring axis duplication

Percentages of axis duplication were obtained by dividing the total number of embryos with a duplicated axis by the total number of embryos, scored from two to six independent experiments combined. Complete axis duplication is defined by the presence of the cement gland and at least one eye in the duplicated axis.

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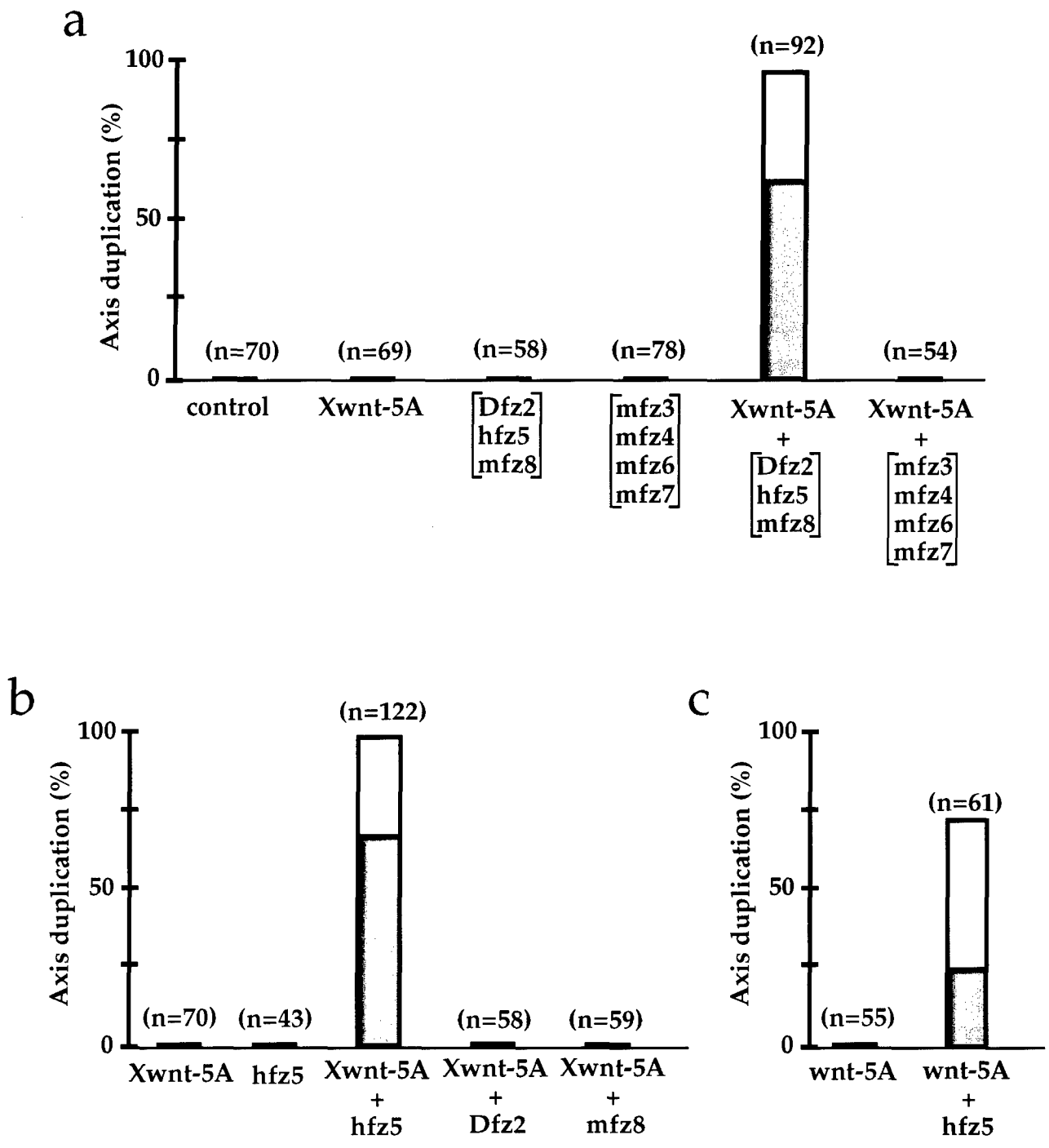
Figure 1 (He et al.)



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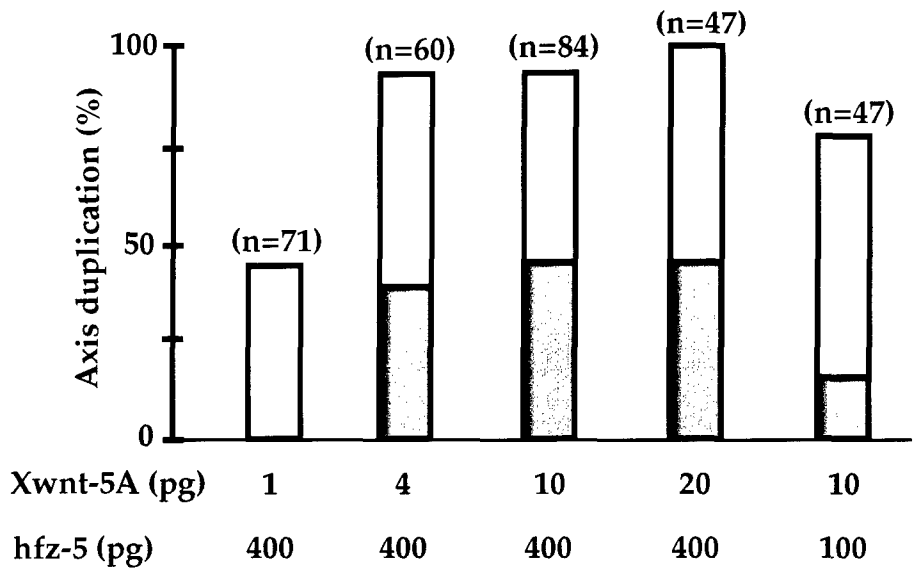

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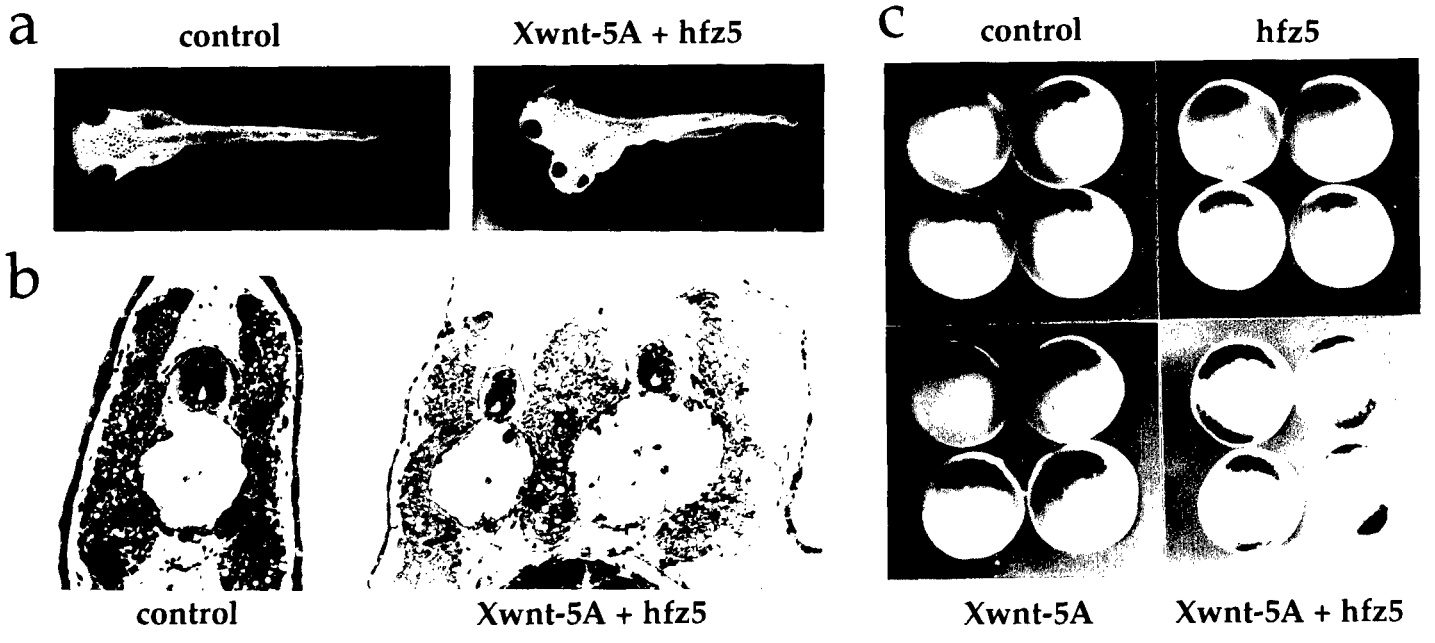

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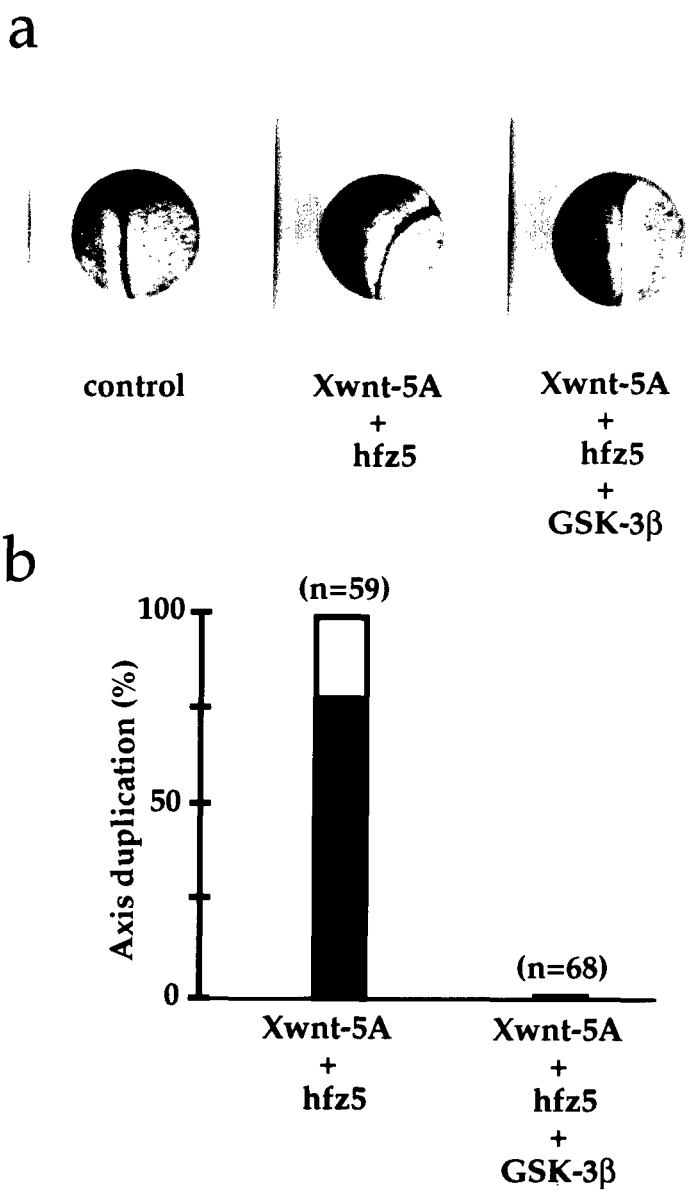

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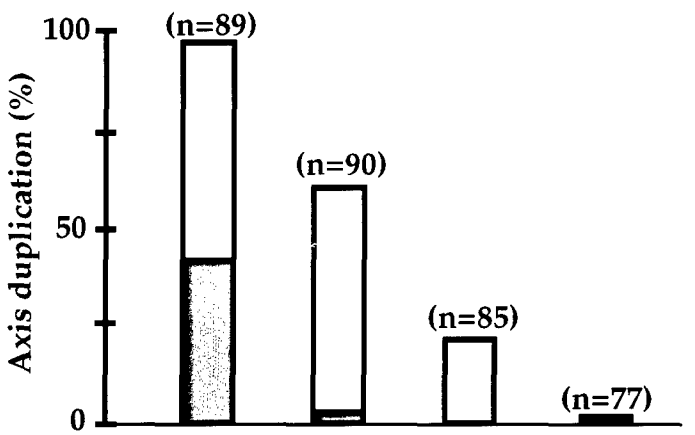

Xwnt-5A (pg)	10	10	10	10
hfz-5 (pg)	400	400	400	400
hfz-5N (pg)	0	400	800	2000

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DAMD17-94-C-4024	ADB189184
DAMD17-94-C-4026	ADB187918
~~DAMD17-94-J-4250~~	~~ADB221970~~
~~DAMD17-94-J-4250~~	~~ADB230700~~
DAMD17-96-1-6241	ADB233224
DAMD17-96-1-6241	ADB218632
DAMD17-94-J-4496	ADB225269
DAMD17-94-J-4392	ADB225308
DAMD17-94-J-4455	ADB225784
DAMD17-94-J-4309	ADB228198
DAMD17-91-C-1135	ADB233658
DAMD17-94-J-4038	ADB232313
DAMD17-94-J-4073	ADB222794
DAMD17-94-J-4131	ADB219168
DAMD17-94-J-4159	ADB232305
MIPR 95MM5535	ADB232218
95MM5605	ADB233374
95MM5673	ADB226037

MCMR-RMI-S
SUBJECT: Request Change in Distribution Statement

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or email: judy_pawlus@ftdetrck-ccmail.army.mil.

FOR THE COMMANDER:

Phyllis Rinehart

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